Z3, a novel Jak2 tyrosine kinase small-molecule inhibitor that suppresses Jak2-mediated pathologic cell growth

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Abstract

Jak2 tyrosine kinase is essential for animal development and hyperkinetic Jak2 function has been linked to a host of human diseases. Control of this pathway using Jak2-specific inhibitors would therefore potentially serve as a useful research tool and/or therapeutic agent. Here, we used a high-throughput program called DOCK to predict the ability of 20,000 small molecules to interact with a structural pocket adjacent to the ATP-binding site of murine Jak2. One small molecule, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one (herein designated as Z3), bound to Jak2 with a favorable energy score. Z3 inhibited Jak2-V617F and Jakt2-WT autophosphorylation in a dose-dependent manner but was not cytotoxic to cells at concentrations that inhibited kinase activity. Z3 selectively inhibited Jak2 kinase function with no effect on Tyk2 or c-Src kinase function. Z3 significantly inhibited proliferation of the Jak2-V617F-expressing, human erythroleukemia cell line, HEL 92.1.7. The Z3-mediated reduction in cell proliferation correlated with reduced Jak2 and STAT3 tyrosine phosphorylation levels as well as marked cell cycle arrest. Finally, Z3 inhibited the growth of hematopoietic progenitor cells isolated from the bone marrow of an essential thrombocythemia patient harboring the Jak2-V617F mutation and a polycythemia vera patient carrying a Jak2-F537I mutation. Collectively, the data suggest that Z3 is a novel specific inhibitor of Jak2 tyrosine kinase. [Mol Cancer Ther 2008;7(8):2308–18]

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Materials and Methods

Small-Molecule Database

The small molecules were obtained from the National Cancer Institute/Developmental Therapeutics Program, which maintains a repository of ~140,000 compounds.

Introduction

Aside from its essential role in embryonic development (1), Jak2 plays an important role in pathophysiology. Jak2 has been linked to several hematologic malignancies including acute lymphoid leukemia and chronic myeloid leukemia (2–4). Recently, a Jak2-V617F somatic mutation has been identified in ~98% of patients with polycythemia vera and in a substantial proportion (50%) of patients with essential thrombocythemia and primary myelofibrosis (5). This valine to phenylalanine substitution (V617F) occurs in the JH2 pseudokinase domain of Jak2. These myeloproliferative disorders are characterized by the clonal overproduction of normally differentiated hematopoietic lineages. The V617F substitution leads to constitutive activation of Jak2 and downstream effector signaling pathways including the STAT transcription pathway and the phosphatidylinositol 3-kinase and extracellular signal-regulated kinase signaling networks, which in turn induce inappropriate cytokine-independent proliferation of cells (5, 6). In addition, the Jak2-V617F mutation contributes to myelofibrosis by constitutively phosphorylating STAT3 and diminishing myeloid cell apoptosis (7). Collectively, these results implicate dysregulated Jak2 signaling in various hematologic and myeloproliferative disorders.

As components of the Jak-STAT signaling pathway are hyperactivated in some leukemias and myeloproliferative disorders, control of this pathway using pharmacologic inhibition is highly desirable. As a research tool, AG490 is by far the most extensively used commercially available Jak2 inhibitor. Although it has been valuable in identifying Jak2 as a therapeutic target, it suffers from a general lack of specificity. For example, AG490 inhibits calf serum-inducible cell growth and DNA synthesis (8) and is a partial blocker of c-Src activity (9). Most importantly, AG490 inhibits epidermal growth factor receptor autophosphorylation more potently than it inhibits Jak2 activity (10, 11). Thus, identification and characterization of novel Jak2-specific inhibitors may serve as useful research tools and/or therapeutic agents.

Here, we used a rapid structure-based approach combining molecular docking with cell-based functional testing to identify a Jak2-selective inhibitor. One small molecule, 2-methyl-1-phenyl-4-pyridin-2-yl-2-(2-pyridin-2-ylethyl)butan-1-one (herein designated as Z3), bound to a pocket that was adjacent to the ATP-binding site of Jak2 with a favorable energy score. Our functional testing data suggest that Z3 is a novel Jak2 tyrosine kinase-specific, small-molecule inhibitor.

Materials and Methods

Small-Molecule Database

The small molecules were obtained from the National Cancer Institute/Developmental Therapeutics Program, which maintains a repository of ~140,000 compounds.
Cell Culture
BSC-40 and COS-7 cells were maintained as described previously (12). Human erythroleukemia (HEL) and Burkitt’s lymphoma (Raji) cells were purchased from the American Type Culture Collection. HEL and Raji cells were maintained in RPMI 1640 (Mediatech) containing 10% fetal bovine serum and 1–g-glutamine (2 mmol/L final). All cells were cultured at 37°C in a 5% CO2 humidified atmosphere.

BSC-40 Cell Transfection/Infection
Jak2 autophosphorylation assays were done in BSC-40 cells using the vaccinia virus transfection/infection procedure, which results in high-level Jak2 expression and subsequent tyrosine phosphorylation independent of exogenous ligand addition (13). Specifically, cells were transfected with 10 μg of a plasmid encoding either the wild-type murine Jak2 cDNA (pRC-CMV-Jak2-WT) or V617F mutant murine Jak2 cDNA (pRC-CMV-Jak2-V617F) under the control of the bacteriophage T7 promoter using Lipofectin per the manufacturer’s instructions (Invitrogen). After 4 h of transfection, the cells were infected with the recombinant vaccinia virus, vTF7-3, at a multiplicity of infection of 1.0 for 1 h. The medium containing Lipofectin/DNA/vTF7-3 was then removed from the cells, replaced with fresh serum-containing medium, and incubated overnight.

COS-7 Cell Transient Transfection
COS-7 cells were transfected with 10 μg of plasmid encoding the wild-type human Tyk2 cDNA (pMT2T-Tyk2-WT) for 5 h using Lipofectin. Following transfection, the cells were grown at 37°C for 48 h in serum-containing medium to allow for high-level Tyk2 tyrosine autophosphorylation. The cells were then treated with DMSO or Z3 for 16 h before lysis.

Cell Lysis and Immunoprecipitation
Cells were washed with 2 volumes of ice-cold PBS containing 1 mmol/L Na3VO4. BSC-40 cells were lysed in 0.8 mL ice-cold radioimmunoprecipitation assay buffer [20 mmol/L Tris (pH 7.5), 10% glycerol, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 2.5 mmol/L EDTA, 50 mmol/L NaF, 10 mmol/L Na2P2O7, 4 mmol/L benzamidine, and 10 μg/mL aprotinin], whereas HEL cells (1.0 × 107) were lysed in 0.8 mL ice-cold gentle lysis buffer [25 mmol/L Tris (pH 7.4), 10% glycerol, 1% IGEPA, and 137 mmol/L NaCl, 4 mmol/L benzamidine, and 10 μg/mL aprotinin]. Cleared protein lysates were incubated with 2 μg antibody and 20 μL protein A/G Plus agarose beads (Santa Cruz Biotechnology) for 2 h at 4°C. Protein complexes were washed three times with wash buffer [25 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, and 0.1% Triton X-100] and resuspended in SDS sample buffer. Bound proteins were boiled, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. The immunoprecipitating anti-Jak2, anti-STAT3, and anti-Tyk2 polyclonal antibodies were from Santa Cruz Biotechnology.

Western Blotting
Anti-phosphotyrosine Western blotting was done using a combination of PY99, PY20, and 4G10 monoclonal antibodies from Santa Cruz Biotechnology, BD Transduction Labs, and Millipore, respectively. Anti-Jak2 pY1007/pY1008 and phospho-STAT3 Western blotting antibodies were from Santa Cruz Biotechnology. The anti-c-Src pY418 was from BioSource. Western blotting for total c-Src protein was done using a cocktail of polyclonal antibodies from Santa Cruz Biotechnology and BD Transduction Laboratories. The anti-Jak2 Western blotting antibodies consisted of a cocktail of antibodies from Millipore and BioSource. Western blots were visualized using the enhanced chemiluminescence system (NEN Life Science Products). Densitometry was done using the automated digitizing software (Un-Scan-It version 5.1; Silk Scientific). All phosphorylation levels were normalized to total protein levels.

c-Src In vitro Kinase Assay
Catalytically active recombinant pp60c-Src [ ~4 μL (12 units); Millipore] was incubated in 50 μL in vitro kinase reaction buffer as described (13). Reactions were terminated via the addition of SDS-containing sample buffer and subsequently Western blotted with either anti-active c-Src or total c-Src antibody as described.

Propidium Iodide Staining of Cells
BSC-40 cells were seeded in six-well Lab-Tek Chamber Slides (Nunc). After adherence, the cells were treated with either DMSO or Z3 at concentrations of 25, 100, or 250 μmol/L for 16 h. Live cells were treated with 1 μg/mL propidium iodide and then visualized using a Bio-Rad MRC-1024 confocal microscope. Same-field images were captured under phase-contrast and fluorescent conditions.

HEL Cell Proliferation
HEL cells were plated on 96-well dishes at 5 × 104 per well and treated with either DMSO or 25 μmol/L Z3 for the indicated times. The number of viable DMSO or Z3-treated cells were determined by trypan blue exclusion using a hemocytometer.

Cell Cycle Analysis
The CycleTEST Plus DNA Reagent Kit (Becton Dickinson) was used to analyze nuclear DNA from HEL cell suspensions following the manufacturer’s instructions. Cells (~5 × 105) were treated with either DMSO or 25 μmol/L Z3 for 16, 24, 48, and 72 h. Cell suspensions were treated with the reagents stated in the protocol, filtered through a 50 μm nylon mesh, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Jak2 Mutation Analysis
Residual bone marrow aspirates were obtained from patients diagnosed with essential thrombocythemia or polycythemia vera (WHO criteria) following approval by the University of Florida Institutional Review Board (approval no. 428-2006). Patient de-identified mononuclear cells were isolated by separation over Ficoll-Paque (Pharmacia). Genomic DNA was isolated from 106 cells using the Easy-DNA kit (Invitrogen) following the manufacturer’s instructions. Exons 12 and 14 were amplified via PCR using previously published exon-specific primers and annealing conditions (14). Each amplified exon was then subjected to direct DNA sequence analysis.

Colony Formation Assay
Marrow-derived mononuclear cells were washed three times in IMDM and plated at 4 × 105/mL in 1 mL...
methylcellulose medium [0.9% methylcellulose, 30% heat-inactivated FCS, 0.1 mmol/L 2-mercaptoethanol, 0.9% bovine serum albumin, 0.05% NaHCO3, 2 mmol/L L-glutamine, penicillin, streptomycin, 50 ng/mL stem cell factor, and 20 ng/mL interleukin-3 (Stem Cell Technologies)]. Z3 (25 μmol/L) and thrombopoietin (50 ng/mL) or erythropoietin (1 units/mL) were either added or omitted as described in the legend. The cultures were then incubated at 37°C and 5% CO2 until assessment of colony formation at day 14. Results were expressed as the average number of colonies from duplicate cultures per 4 × 105 cells.

Results

Z3 Inhibits Jak2 Tyrosine Autophosphorylation by Interacting with a Solvent-Accessible Pocket Adjacent to the ATP-Binding Pocket

Taking the crystal structure for portions of the Jak3 kinase domain (PDB code 1YVJ) into consideration, a comparative structural modeling approach was employed to generate an atomic model of the kinase domain of murine Jak2 using ProModII as described previously (12).

The program SPHGEN, which identifies potential ligand-binding sites based on chemical and shape characteristics, was employed to select the target pocket on Jak2 for small-molecule docking. To prepare the site for docking, all water molecules were removed. Protonation of receptor residues was done with the program Sybyl. The sphere set used for molecular docking was based on the position of a Jak3 inhibitor in the crystal structure of the kinase domain of Jak3 (Fig. 1A). The position of a pan-Jak2 inhibitor that was subsequently crystallized in the kinase domain of human Jak2 is also shown (Fig. 1A). The site selected for molecular docking was adjacent to the ATP-binding site of murine Jak2 and the number of spheres in the site (50-100) was in the ideal range for binding to small molecules.

Using the DOCK program, we tested 20,000 compounds of known chemical structure, in silico, for their ability to interact with a structural pocket adjacent to the ATP-binding site. The top six scoring compounds (Supplementary Fig. S1)3 were obtained from the National Cancer Institute/Developmental Therapeutics Program and tested for their ability to inhibit Jak2-WT and Jak2-V617F autophosphorylation.

A Jak2 overexpression system was used to first determine the effect of each inhibitor on Jak2 autophosphorylation. Specifically, BSC-40 cells were transfected with an expression vector encoding either empty vector control (no Jak2), the mutant murine Jak2 cDNA (Jak2-V617F), or the wild-type Jak2 cDNA (Jak2-WT) under the control of T7 RNA polymerase. This resulted in high-level Jak2 expression and subsequent tyrosine phosphorylation independent of exogenous ligand addition. The six inhibitor compounds (Z1-Z6) were each added at a concentration of 100 μmol/L and incubated overnight. Sixteen hours following addition of the compounds, cellular lysates were immunoprecipitated with an anti-Jak2 polyclonal antibody and then immunoblotted with an anti-phosphotyrosine monoclonal antibody to detect tyrosine-phosphorylated Jak2 (Fig. 1B, top). The results show that compound Z3 most effectively inhibited both Jak2-V617F and Jak2-WT tyrosine autophosphorylation. Clearly however, the ability of Z3 to inhibit Jak2-WT autophosphorylation was greater than when compared with Jak2-V617F autophosphorylation. The membrane was then stripped and reprobed with an anti-Jak2 polyclonal antibody to show total protein loading (Fig. 1B, bottom).

The structure of the Z3 compound is shown in Fig. 1C. Based on the data in Fig. 1B, we returned to our molecular model of Jak2 and used the DOCK program to determine the position of Z3 on the Jak2 structure. Based on contact points and energy scores, the Z3 inhibitor was predicted to bind to a solvent-accessible pocket adjacent to the ATP-binding site as we had intended (Fig. 1D). Collectively, the data in Fig. 1 indicate that Z3 inhibits both Jak2-WT and Jak2-V617F tyrosine autophosphorylation (kinase function) via an interaction with a critical solvent-accessible pocket adjacent to the ATP-binding site.

Z3 Inhibits Jak2 Tyrosine Autophosphorylation in a Dose-Dependent Manner That Is Independent of Cellular Cytotoxicity

To obtain a better understanding of the inhibitory properties of Z3, we examined whether it could inhibit Jak2-WT and Jak2-V617F tyrosine autophosphorylation in a dose-dependent manner. Specifically, we sought to determine whether Z3 inhibits phosphorylation of the critically important Tyr1007 residue, as phosphorylation of this residue within the activation loop of Jak2 is necessary for maximal Jak2 activation (15). To meet this end, BSC-40 cells were transfected/infected with either a Jak2-WT or a Jak2-V617F plasmid and then treated for 16 h with Z3 at the indicated concentrations. For the Jak2-WT-expressing cells, cell lysates were first immunoprecipitated with a Jak2 polyclonal antibody and then Western blotted with a phosphospecific (pY1007/pY1008) Jak2 antibody to detect phosphorylated Jak2 at this residue (Fig. 2A, top). The results indicate that Z3 inhibited phosphorylation of the critically important Tyr1007 residue on Jak2-WT in a dose-dependent manner. The membrane was subsequently stripped and reprobed with an anti-Jak2 polyclonal antibody to determine Jak2 expression levels among all samples (Fig. 2A, bottom).

To quantitate Jak2-WT Tyr1007 phosphorylation levels in the presence of increasing amounts of Z3, densitometry analysis on four representative Western blots was done (Fig. 2B). We found that Jak2-WT tyrosine autophosphorylation was reduced by ~60% between 10 and 30 μmol/L. A 100 μmol/L dose of Z3 maximally suppressed Jak2 phosphorylation by ~92%. The estimated IC50 of Z3 for Jak2-WT was 15 μmol/L.

3 Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
For the Jak2-V617F samples, Jak2 immunoprecipitates were immunoblotted with the same pY1007/pY1008 phosphospecific antibody (Fig. 2C, top). Similar to the Jak2-WT-expressing cells, Z3 inhibited Jak2-V617F Tyr1007 phosphorylation in a dose-dependent fashion. The membrane was then stripped and reprobed with an anti-Jak2 antibody to determine relative Jak2 expression across all samples (Fig. 2C, bottom). Densitometry analysis of four representative Western blots showed that Jak2-V617F Tyr1007 phosphorylation was inhibited by ~60% between the concentrations of 10 and 100 μmol/L Z3 (Fig. 2D). The IC_{50} of Z3 for Jak2-V617F was ～28 μmol/L.

To rule out the possibility that the effects of Z3 were due to nonspecific cellular toxicity rather than specific Jak2 inhibition, we treated these same BSC-40 cells with either DMSO or 25, 100, or 250 μmol/L Z3 for 16 h. The live cells were then stained with propidium iodide. Propidium iodide selectively stains necrotic cells and fluoresces red but is excluded from the plasma membrane of intact cells. The results show that cells treated with 25 or 100 μmol/L Z3 showed propidium iodide staining similar to that of DMSO-treated cells (Supplementary Fig. S2). How-ever, cells treated with 250 μmol/L Z3 displayed a marked increase in propidium iodide staining, indicating that this dose of Z3 is cytotoxic. The same propidium iodide fields were also visualized by phase-contrast microscopy to determine total cell numbers and overall cellular morphology (Supplementary Fig. S2, bottom). Overall, the data indicate that the range of Z3 that inhibits Jak2 tyrosine kinase autophosphorylation by 50% to 100% (25-100 μmol/L) does so in a manner that is independent of gross cellular cytotoxicity. Taken together, our results indicate that the Z3 compound blocks Jak2 autophosphorylation in a dose-dependent manner but is not cytotoxic to cells at concentrations that inhibit Jak2 tyrosine kinase activity. Moreover, the ability of Z3 to inhibit Jak2-WT (IC_{50} ～15 μmol/L) is greater when compared with Jak2-V617F (IC_{50} ～28 μmol/L).

**Z3 Is a Specific Inhibitor of Jak2 Tyrosine Kinase**

To determine whether Z3 is selective for suppressing Jak2 autophosphorylation when compared with other Jak family members, we employed an autophosphorylation assay to allow for robust Jak family kinase activation independent of ligand treatment (13). Here, COS-7 cells were transiently

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Figure 1. Z3 inhibits Jak2-V617F and Jak2-WT tyrosine autophosphorylation. A, the sphere set used for molecular docking was based on the position of a solvent-accessible pocket adjacent to the Jak2 activation loop. For reference, the positions of the resolved structures for portions of both Jak2 and Jak3 kinase domains are indicated. Also shown is the position of the IZA pan-Jak2 inhibitor that was crystallized within the Jak2 kinase domain. B, BSC-40 cells were transfected with empty vector control, Jak2-V617F, or Jak2-WT expression plasmids and then infected with vaccinia virus to drive high-level expression and subsequent Jak2 tyrosine autophosphorylation. The six highest scoring compounds identified by DOCK were incubated with the cells at a concentration of 100 μmol/L each for 16 h. Cell lysates were immunoprecipitated with anti-Jak2 antibody and immunoblotted with anti-phosphotyrosine antibody to detect Jak2 tyrosine phosphorylation levels (top). The membrane was stripped and reprobed with anti-Jak2 antibody to show equal Jak2 expression among all samples (bottom). One of two independent experiments. C, molecular structure of the Z3 compound. D, based on contact points and energy scores, Z3 docks into a structural pocket on the Jak2 kinase domain that is adjacent to the ATP-binding site.
transfected with an expression vector encoding the wild-type human Tyk2 cDNA. The following day, the cells were treated with either DMSO or 25 μmol/L Z3 for 16 h. The cells were then lysed and Tyk2 was immunoprecipitated from the lysates via an anti-Tyk2 antibody. The immunoprecipitated protein was then immunoblotted with an anti-phosphotyrosine antibody to evaluate the level of Tyk2 tyrosine autophosphorylation (catalytic activity). We found that Z3 did not inhibit Tyk2 tyrosine kinase autophosphorylation when compared with DMSO control (Fig. 3A, top), bearing in mind that this same 25 μmol/L dose reduced Jak2-WT tyrosine kinase autophosphorylation by ~70% (Fig. 2B). The membrane was subsequently stripped and reprobed with anti-Tyk2 antibody to show equal Tyk2 expression for both samples (Fig. 3A, bottom).

We next investigated the effect of Z3 on another nonreceptor tyrosine kinase, c-Src. Similar to Jak2, c-Src is ubiquitously expressed and has been shown to activate several effectors that are also involved in aberrant cell growth. Here, catalytically active recombinant c-Src protein was incubated either in the presence of DMSO, 25 μmol/L Z3, or 25 μmol/L of the Src kinase inhibitor, PP2. The reactions were incubated for 20 min at room temperature and then terminated by addition of SDS-containing sample buffer. The samples were separated by SDS-PAGE and subsequently Western blotted with an anti-active c-Src (pY418) antibody to determine c-Src catalytic activity (Fig. 3B, top). We found that Z3 had no effect on c-Src tyrosine kinase activity when compared with DMSO control. However, in the presence of the known c-Src

Figure 2. Z3 inhibits Jak2 tyrosine autophosphorylation in a dose-dependent manner. A, BSC-40 cells were transfected/infected as described above. Jak2-WT-expressing cells were incubated with either vehicle control (DMSO) or with Z3 at the indicated doses. Protein cell lysates were then immunoprecipitated with anti-Jak2 antibody and immunoblotted with a phosphospecific (pY1007/pY1008) Jak2 antibody to detect phosphorylated Jak2 at this specific residue (top). The membrane was stripped and reprobed with anti-Jak2 antibody to show equal Jak2 expression among all samples (bottom). B, densitometric analysis was done on four representative Western blots to quantify Z3-mediated inhibition of WT-Jak2 phosphorylation at the Tyr1007 residue. Mean ± SE ratio of phosphorylated Jak2 to total Jak2. The IC50 of Z3 for Jak2-WT was ~15 μmol/L. Statistical significance between the vehicle control and Z3-treated cells was determined by a one-way ANOVA (P < 0.001). C, for Jak2-V617F expression, cells were first immunoprecipitated with a Jak2 polyclonal antibody and then Western blotted with a phosphospecific (pY1007/pY1008) Jak2 antibody (top). The membrane was then stripped and reprobed with anti-Jak2 antibody (bottom). D, densitometry analysis of four representative Western blots showing Z3-mediated inhibition of Jak2-V617F phosphorylation at the Tyr1007 residue. Mean ± SE ratio of phosphorylated Jak2 to total Jak2. The IC50 of Z3 for Jak2-V617F was ~28 μmol/L. There was a statistically significant difference between the vehicle control and Z3-treated cells (P < 0.001).
inhibitor, PP2, c-Src tyrosine kinase activity was completely abolished. To show equal c-Src protein content among all conditions, the membrane was stripped and reprobed with anti-c-Src antibody (Fig. 3B, bottom). Collectively, the results show that although Z3 suppresses Jak2 tyrosine kinase activity, it does not inhibit Tyk2 or c-Src.

Z3 Selectively Inhibits Jak2-V617F-Dependent Cell Proliferation and This Correlates with Suppression of Jak2 and STAT3 Tyrosine Phosphorylation

It is known that the human erythroleukemia cell line, termed HEL, is homozygous for the Jak2-V617F mutation (16). The single point mutation leading to a V617F substitution in the JH2 domain of Jak2 has been associated with its proliferative phenotype (16). In addition, it has been shown that expression of the constitutively activated Jak2-V617F mutation is required for transformation of HEL cells. Mechanistically, the Jak2-V617F mutation promotes G1-S-phase transition in HEL cells and subsequent increases cellular proliferation (17).

Due to the presence of the constitutively activated Jak2-V617F mutation in HEL cells, we wanted to determine whether Z3 could suppress Jak2-V617F-dependent cell proliferation in this cell line. Here, 5 x 10^4 HEL cells were treated with either DMSO or 25 μmol/L Z3 for 0, 16, 24, 48, and 72 h. At the end of each time point, the number of viable DMSO- or Z3-treated cells was determined by trypan blue exclusion using a hemocytometer (Fig. 4A). The results show that, in the presence of DMSO, HEL cells were in rapid growth. However, treatment with Z3 reduced cell numbers when compared with DMSO. ANOVA indicated that the Z3 growth curve was significantly different from that of DMSO (P = 1.06 x 10^-14).

To determine whether Z3 specifically suppresses Jak2-V617F-dependent cell growth, as opposed to nonspecific inhibition of cell proliferation, we examined the inhibitory effect of Z3 on Raji cells. The molecular mechanism responsible for the aberrant growth of Raji cells is a translocation event between the c-Myc gene on chromosome 8 and the heavy chain locus on chromosome 14 (18). Here, 5 x 10^3 HEL or Raji cells were treated for 72 h with either DMSO or Z3 at the indicated concentrations. The number of viable cells was then determined (Fig. 4B). The results indicate that HEL cells were more sensitive to the inhibitory effects of Z3, over a wide range of doses, when compared with Raji cells (P = 5.28 x 10^-11).

We next examined whether Z3-dependent inhibition of HEL cell proliferation correlated with suppression of Jak2 tyrosine autophosphorylation. Specifically, HEL cells were treated with either DMSO or 25 μmol/L Z3 for the indicated times. Whole-cell protein lysates were Western blotted with the phosphospecific (pY1007/Jak2) antibody to detect total Jak2 tyrosine-phosphorylated Jak2 (Fig. 4C, top). We found that Z3 dramatically reduced total Jak2 tyrosine autophosphorylation by 48 h when compared with DMSO control and this correlated with inhibition of HEL cell proliferation at this time point. The membrane was then stripped and reprobed with anti-Jak2 antibody to show total Jak2 expression among all samples (Fig. 4C, bottom).

We next sought to determine whether Z3 could inhibit Jak2 phosphorylation at Tyr1007 in these same cells. After treatment of the cells with either DMSO or 25 μmol/L Z3 for the indicated times, whole-cell protein lysates were Western blotted with the phosphospecific (pY1007/Jak2) antibody.
pY1008) Jak2 antibody to detect phosphorylated Jak2 at this residue (Supplementary Fig. S3, top). The results show that Z3 inhibited Jak2 phosphorylation at Tyr1007 in a time-dependent manner when compared with DMSO control. The membrane was subsequently stripped and reprobed with anti-Jak2 antibody to show total Jak2 protein expression among all samples (Supplementary Fig. S3, bottom).

STAT3 is a known substrate of Jak2 and previous studies have shown that STAT3 is constitutively phosphorylated in HEL cells (19, 20). We therefore examined whether Z3-mediated inhibition of Jak2-dependent cell proliferation in HEL cells also correlates with reduced STAT3 phosphorylation. Specifically, protein cell lysates were immunoprecipitated with a STAT3 antibody and then immunoblotted with a phosphospecific (pY705) STAT3 antibody to assess active STAT3 levels (Fig. 4D, top). We found that Z3 inhibited STAT3 tyrosine phosphorylation in HEL cells when compared with DMSO control, thus showing that reduced phospho-STAT3 levels also correlate with reduced cell numbers. The membrane was stripped and reprobed with a STAT3 antibody to show equal STAT3 content among all samples (Fig. 4D, bottom).

Overall, the data in Fig. 4 show that Z3 selectively blocks Jak2-V617F pathologic cell growth and this corresponds with reduced levels of activated Jak2 and STAT3.

Z3 Exerts Its Effect on the Cell Cycle by Increasing the Percentage of HEL Cells in G1 Phase while Decreasing the Number of Cells in S Phase

To determine the mechanism of Z3-mediated inhibition in cell growth, we first examined whether treatment with Z3 corresponded to an increase in apoptosis in the HEL cells. Specifically, HEL cells were treated with either DMSO or 25 μmol/L Z3 for 16, 24, 48, or 72 h and cells were measured for Annexin V staining via flow cytometry. Treatment of cells with Z3 did not result in a significant increase in the percentage of Annexin V–positive cells when compared with DMSO-treated cells (data not shown), indicating that Z3 did not induce apoptosis in this cell type. In addition, HEL cells treated with 25 μmol/L Z3 stained negative for an early marker of apoptosis, APO2.7.

Figure 4. Z3 selectively suppresses Jak2-dependent cell proliferation, which correlates with inhibition of Jak2 and STAT3 phosphorylation. A, HEL cells were treated with either DMSO or 25 μmol/L Z3 for 0, 16, 24, 48, or 72 h. The numbers of viable DMSO- or Z3-treated cells were assessed by trypan blue exclusion. All data points were measured in triplicate. Statistical significance between each group was analyzed using a two-way ANOVA. The two conditions were found to be significantly different ($P = 1.06 \times 10^{-14}$). B, HEL and Raji cells were treated with either DMSO or 0.1, 0.3, 1, 3, 10, or 30 μmol/L Z3. After 72 h, the numbers of viable cells were assessed as described above. The graph shown is the compilation of three independent experiments. Statistical significance between each group was analyzed using a two-way ANOVA. The two conditions were found to be significantly different ($P = 5.28 \times 10^{-11}$). C, HEL cells were treated with either DMSO or 25 μmol/L Z3 for 16, 24, 48, or 72 h. Protein lysates were immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-phosphotyrosine antibody to determine total Jak2 phosphorylation levels (top). The membrane was stripped and reprobed with Jak2 antibody to show Jak2 expression among all samples (bottom). D, HEL cells were treated as indicated. STAT3 phosphorylation levels were determined by first immunoprecipitating with STAT3 antibody and then immunoblotting with a phosphospecific (pY705) STAT3 antibody (top). The membrane was stripped and reprobed with STAT3 to show total STAT3 protein content among all samples (bottom). One of three (A and B) or two (C and D) representative experiments.
confirming that the mechanism by which Z3 suppresses HEL cell proliferation is independent of apoptosis (data not shown).

We next examined cell cycle distribution as a function of Z3 treatment using the CycleTest PLUS DNA Kit. HEL cells were treated with either DMSO or 25 μmol/L Z3 for 16, 24, 48, or 72 h and cell cycle variables were determined by fluorescence-activated cell sorting analysis. We found that Z3 significantly increased the percentage of cells in the G1 phase (Fig. 5A) and significantly decreased the percentage of cells in S phase (Fig. 5B) when compared with DMSO control-treated cells. A representative experiment of the cell cycle profile for DMSO- and Z3- treated HEL cells is also shown (Fig. 5C and D, respectively). Collectively, the results in Fig. 5 reveal that the mechanism by which Z3 reduces cell numbers is by blocking G1-S transition.

Z3 Reduces Hematopoietic Colony Formation Ex vivo

Somatic Jak2 mutations have been described in myeloproliferative disorders (14, 21, 22). The most common Jak2 mutation, Jak2-V617F, has been shown to be the cause of several myeloproliferative diseases including polycythemia vera, essential thrombocythemia, and primary myelofibrosis. We have already shown that Z3 suppresses Jak2-dependent cell growth in the HEL cell line (Fig. 4A). We next turned our attention to determining whether Z3 could inhibit the growth of hematopoietic progenitor cells isolated from the bone marrow of confirmed myeloproliferative disorder patients. Here, residual bone marrow aspirates were obtained from an essential thrombocythemia patient who was Jak2-V617F positive (Fig. 6A) or from a polycythemia vera patient who was Jak2-F537I positive (Fig. 6B). Their hematopoietic progenitor cells were cultured in a semisolid colony assay medium in the presence of DMSO or 25 μmol/L Z3. In addition, because hematopoietic progenitors taken from patients with myeloproliferative disorders are known to be hypersensitive to cytokine stimulation (23), the cells taken from the essential thrombocythemia patient were cultured in both the presence and the absence of human thrombopoietin, whereas the cells taken from the polycythemia vera patient were cultured in the presence and absence of human erythropoietin. The results show that, as expected, treatment of the essential thrombocythemia patient’s hematopoietic progenitor cells with thrombopoietin markedly increased

![Figure 5](image_url)

**Figure 5.** Z3 induces cell cycle arrest in Jak2-V617F-transformed human erythroleukemia cells. HEL cells were treated with either DMSO or 25 μmol/L Z3 for 16, 24, 48, or 72 h and cell cycle effects were determined. FACSCalibur flow cytometer along with ModFit software (Verity Software) was used to analyze DNA contents. The mean and SD of samples were determined. Statistical significance between each group was analyzed using a two-way ANOVA. A, percentage of HEL cells in G1 phase following treatment with either DMSO or Z3 for the indicated times. The two conditions were considered to be significantly different (P = 0.000407). B, percentage of HEL cells in S phase following treatment with either DMSO or Z3 for the indicated times. The two conditions were considered to be significantly different (P = 0.0256). Representative of three independent experiments (A and B). Representative cell cycle analysis profile from one of those three experiments after 72 h treatment with either DMSO (C) or Z3 (D).
megakaryocyte colony formation (Fig. 6A). However, this cytokine-dependent increase in cell growth was significantly blunted when the cells were cultured in the presence of Z3. Similarly, treatment of the polycythemia vera patient’s progenitor cells with erythropoietin significantly increased erythrocyte colony formation. However, treatment of the cell with Z3 significantly reduced both cytokine-independent and cytokine-dependent erythrocyte colony formation (Fig. 6B). Collectively, the results in Fig. 6 show that Z3 greatly reduces Jak2-V617F as well as Jak2-F537I-mediated, human pathologic cell growth ex vivo.

Discussion

Hyperkinetic Jak2 tyrosine kinase activity has been linked to a variety of human diseases including cardiovascular disease, diabetes, and cancer (24–28). In recent years, the Jak2-V617F gain-of-function mutation in myeloproliferative disorders has also been well described (5–7, 29, 30). Mutations in exon 12 of Jak2, which cause dysregulated Jak-STAT signaling, have been identified in Jak2-V617F-negative myeloproliferative disorders as well (14, 31, 32). Thus, the continued identification of novel agents that can inhibit aberrant Jak2 tyrosine kinase function will be of great value.

Here, we have used in silico homology modeling and high-throughput molecular docking to identify a novel small-molecule inhibitor of Jak2. Our results are significant for several reasons. First, Z3 inhibits Jak2-WT and Jak2-V617F autophosphorylation but is not cytotoxic to cells at concentrations that inhibit Jak2. Second, Z3 inhibits phosphorylation of Tyr1007, a residue whose phosphorylation is concomitant with hyperkinetic Jak2 function. Third, Z3 selectively inhibits Jak2 tyrosine kinase activity as it has no effect of Tyk2 and c-Src. Fourth, Z3 preferentially suppresses proliferation of HEL cells that express the Jak2-V617F mutation by inducing cell cycle arrest. This arrest in cell growth directly correlates with reduced levels of active Jak2 and STAT3 proteins. Fifth, Z3 significantly blocks the growth of hematopoietic progenitor cells isolated from the bone marrow of myeloproliferative disorder patients carrying Jak2 mutations.

The Z3 compound is yet another step in the continuing development of Jak2 inhibitors. High-throughput screening of potential tyrosine kinase inhibitors identified tyrphostin B42 (AG490) as the first Jak2 inhibitor. Initially, AG490 was regarded as a specific inhibitor of Jak2 because it concomitantly inhibited Jak2 tyrosine phosphorylation levels and suppressed acute lymphoblastic leukemia by inducing cellular apoptosis (33). However, other studies suggested that it suffered from a general lack of specificity (9–11). Ensuing work by multiple groups, including our own, has identified various small molecules or, in some cases, a protein mimetic that block Jak2 kinase function (12, 34–36). Despite the fact that these agents all inhibit Jak2 kinase function, differences in both the structure and the mechanism of action of the inhibitors is striking. For example, AG490 inhibits Jak2 and promotes cellular apoptosis (33), whereas our Z3 compound inhibits Jak2 and promotes cell cycle arrest. Interestingly, the TG101209 compound recently described by Pardanani et al. inhibits Jak2 and causes both increased apoptosis and cell cycle arrest (34). Finally, work by Flowers et al. characterized a peptide inhibitor of Jak2 that mimics the Jak2 inhibitory protein, SOCS-1 (36). The peptide mimetic was designed to recognize this

Figure 6. Z3 suppresses Jak2-mediated hematopoietic colony formation ex vivo. **A**, marrow-derived mononuclear cells from an essential thrombocythemia patient who was Jak2-V617F positive were cultured in methylcellulose medium containing either DMSO (0.25%, v/v) or 25 μmol/L Z3. The number of megakaryocyte colonies was assessed 14 d later. Results are expressed as the average number of colonies from duplicate cultures per 4 × 105 cells. Statistical significance between each group was analyzed using Student’s t test. *, P = 0.017. **B**, marrow-derived mononuclear cells from a polycythemia vera patient who was Jak2-F537I positive were cultured in methylcellulose medium containing either DMSO (0.25%, v/v) or 25 μmol/L Z3. The number of erythrocyte colonies was assessed 14 d later. Results are expressed as the average number of colonies from duplicate cultures per 4 × 105 cells. Statistical significance between each group was analyzed using Student’s t test. *, P = 0.0245; **, P = 0.0195.
autophosphorylation site of Jak2 similar to SOCS-1. They found that the peptide mimicked SOCS-1 in that it suppressed Jak2 tyrosine autophosphorylation and subsequent IFN-γ-dependent signaling independent of marked changes in apoptosis or cell cycle progression. Collectively, these works suggest that the relationship between blocking Jak2 kinase function and the eventual fate of the cell is complex and warrants further examination.

Although the Jak2-V617F mutation on exon 14 is the predominant disease-associated allele in myeloproliferative disorders, several other Jak2 exon 14 mutations have been identified. For example, unique C616Y and D620E substitution mutations have been identified in V617F-negative, myeloproliferative disorder individuals (37, 38). Additionally, several mutations have been identified in Jak2 exon 12, including a F537 deletion in one individual and F537 duplication in another (14). Furthermore, chromosomal translocations between the Jak2 allele and other alleles, including TEL, REL, PCM1, and BCR, have all been linked to several hematologic malignancies (2, 39–41). Although each specific translocation gives rise to a unique chimeric protein, they all share one common feature (they all exhibit hyperkinetic Jak2 kinase activity and subsequent malignant hyperplasia). Thus, given the growing number of known Jak2 somatic cell mutations and chromosomal translocations as well as the diverse human diseases that hyperkinetic Jak2 kinase activity has been associated with, identifying inhibitors that can block multiple Jak2 mutations, such as our Z3 compound, will be of great value.

In summary, our results suggest that Z3 inhibits Jak2 tyrosine kinase function. It suppresses Jak2-dependent pathologic cell growth in vitro via a Jak2/STAT3-dependent mechanism that results in cell cycle arrest. Additionally, it blocks ex vivo hematopoietic progenitor cell growth from an essential thrombocytopenia patient who harbors Jak2-V617F and a polycythemia vera patient who carries a novel Jak2-F537I mutation. As such, this compound may have practical applications in Jak2-related research and perhaps serve as a lead therapeutic agent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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